MEDICAL SCIENCE MONITOR

Establishment of an Animal Model of Vascular Restenosis with Bilateral Carotid Artery Grafting

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background:
Vascular restenosis occurring after CABG is a major clinical problem that needs to be addressed. Vein grafts are associated with a higher degree of stenosis than artery grafts. However, the mechanism responsible for this effect has not been elucidated. We aimed to establish a rabbit model of vascular restenosis after bilateral carotid artery grafting, and to investigate the associated spatiotemporal changes of intimal hyperplasia in carotid artery and jugular vein grafts after surgery.

Material/Methods:
Twenty adult New Zealand white rabbits (10 males; 10 females), weighing 2.0–2.5 kg, were obtained from the Experimental Animal Center of Southern Medical University, Guangzhou, China (License No.: scxk-Guangdong-2006-0015). We quantitatively analyzed intimal thickness, area, and degree of stenosis in carotid artery and jugular vein bridges.

Results:
After 8 weeks of a high-fat diet, rabbit carotid arteries showed early atherosclerotic lesions. With increasing time after surgery, carotid artery and jugular vein grafts showed histopathological and morphological changes, including smooth muscle cell migration, lipid deposition, intimal hyperplasia, and vascular stenosis. The degree of vascular stenosis was significantly higher in vein grafts than in artery grafts at all time points – 35.1±6.7% *vs.* 16.1±2.6% at Week 12, 56.2±8.5% *vs.* 23.4±3.4% at Week 16, and 71.2±1.3% *vs.* 25.2±5.3% at Week 20.

Conclusions:
Rabbit bilateral carotid arteries were grafted with carotid artery and jugular vein bridges to simulate pathophysiological processes that occur in people after CABG surgery.

MeSH Keywords:
Graft Occlusion, Vascular • Models, Animal

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Background

Coronary artery bypass grafting (CABG) is the primary treatment option for patients with coronary artery stenosis. Autologous blood vessels are the main source of graft vessels. However, postoperative vascular graft restenosis significantly affects the long-term therapeutic effect of CABG. Studies have shown that the use of saphenous vein grafts for CABG results in rates of postoperative vein graft occlusion of approximately 12–27% in the first year and 25–31% after 5 years. Thereafter, the rate of vein graft occlusion increases annually by 2–4%, and 48–66% of vein grafts are completely occluded by 10 years after surgery [1–4]. In contrast, the patency rate of internal thoracic artery bypass grafts is greater than 85% by 10 years after surgery. By comparison, long-term stenosis rates tend to be significantly lower in arterial grafts than in vein grafts.

Vascular restenosis occurring after CABG is a major clinical problem that needs to be addressed. Vein grafts are associated with a higher degree of stenosis than artery grafts. However, the mechanism responsible for this effect has not been elucidated. Establishment of an animal model that recapitulates vascular restenosis with bilateral carotid artery grafting would be extremely useful for better understanding the pathogenesis of this condition in human patients.

A number of animal models have been developed to study different treatment options for bypass surgery. Rabbits are excellent models for diet-induced hypercholesterolemia and are amenable to performing anastomoses with running sutures. Vein grafts in rabbits are also suitable for testing pharmaceuticals, particularly for the treatment of diabetes, hypertension, or hypercholesterolemia [5,6]. Previously described animal models have typically used lateral carotid artery grafting of a vein or artery bridge. Due to individual differences among animals, application of these existing models might be limited. To address this deficiency, we performed bilateral carotid artery grafting of carotid artery and jugular vein bridges. This approach accurately recapitulates the pathological state of patients following coronary artery bypass grafting (CABG) and, thus, will facilitate research on the mechanism of vascular graft restenosis after CABG.

Material and Methods

Animals, reagents, and materials

Twenty adult New Zealand white rabbits (10 males; 10 females), weighing 2.0–2.5 kg, were obtained from the Experimental Animal Center of Southern Medical University, Guangzhou, China (License No.: scxk-Guangdong-2006-0015). All experimental procedures were approved by the Animal Care Committee of Shantou Central Hospital, Shantou, China. All animals were fed a high-fat diet – standard rabbit feed formulation supplemented with 1% cholesterol, 0.35% cholalic acid, 5% lard, and 0.61% propylthiouracil in addition to high-dose VitD3 (700 000 IU/kg, administered at Day 3). Additional materials included a medical loupe for surgical use and an 8/0 Prolene suture.

Experimental design

The 20 animals were fed a high-fat diet for 8 weeks and then grafted with a carotid artery bridge and a jugular vein bridge in the bilateral carotid arteries. Arterial and venous specimens were obtained during surgery to assess the occurrence of atherosclerotic changes in animals prior to surgery. After the surgical procedure, 1 animal died and another had no atherosclerotic changes upon examination of vascular specimens. The remaining 18 animals were continuously fed a high-fat diet and then sacrificed at Weeks 12, 16, and 20 after surgery (n=6 for each time point). Specimens of artery and vein grafts were obtained to perform pathomorphological analysis to assess the degree of stenosis.

Surgical procedure

Intramuscular anesthesia was administered using 40–50 mg/kg ketamine. A median cervical incision was made in each animal; this incision was then dissected layer-by-layer to expose the trachea. The left and right carotid arteries and jugular veins were identified at both sides of the trachea (Figure 1A).

First, the right carotid artery and jugular vein were freed by 2 cm each and both were closed at the distal and proximal ends using a damage-free vascular clamp. An oblique transverse incision was made in the right carotid artery and a segment of carotid artery (approximately 1 cm in size) was excised for use. The oblique sections at the distal and proximal ends of the carotid artery were sheared into a horn-like shape. Two incisions, approximately 1.5 mm in length, were made in the blocked interval of the right jugular vein parallel to its vertical axis using an arterial blade for CABG surgery. The 2 horn-shaped stumps of the right carotid artery were connected to the right jugular vein via end-to-side anastomosis, followed by continuous suture of anastomotic stomas using an 8/0 Prolene suture. Next, the right carotid artery was released for air exhaust using a 1-mL syringe and then ligated at the distal and proximal ends of anastomotic stomas. In this way, a jugular vein bridge was built to bypass arterial blood flow from the proximal part into the distal part of the carotid artery (Figure 1B).

Second, the left carotid artery was freed by approximately 1 cm and blocked at the distal and proximal ends using a damage-free vascular clamp. An oblique transverse incision was made in the left carotid artery, and the excised right carotid
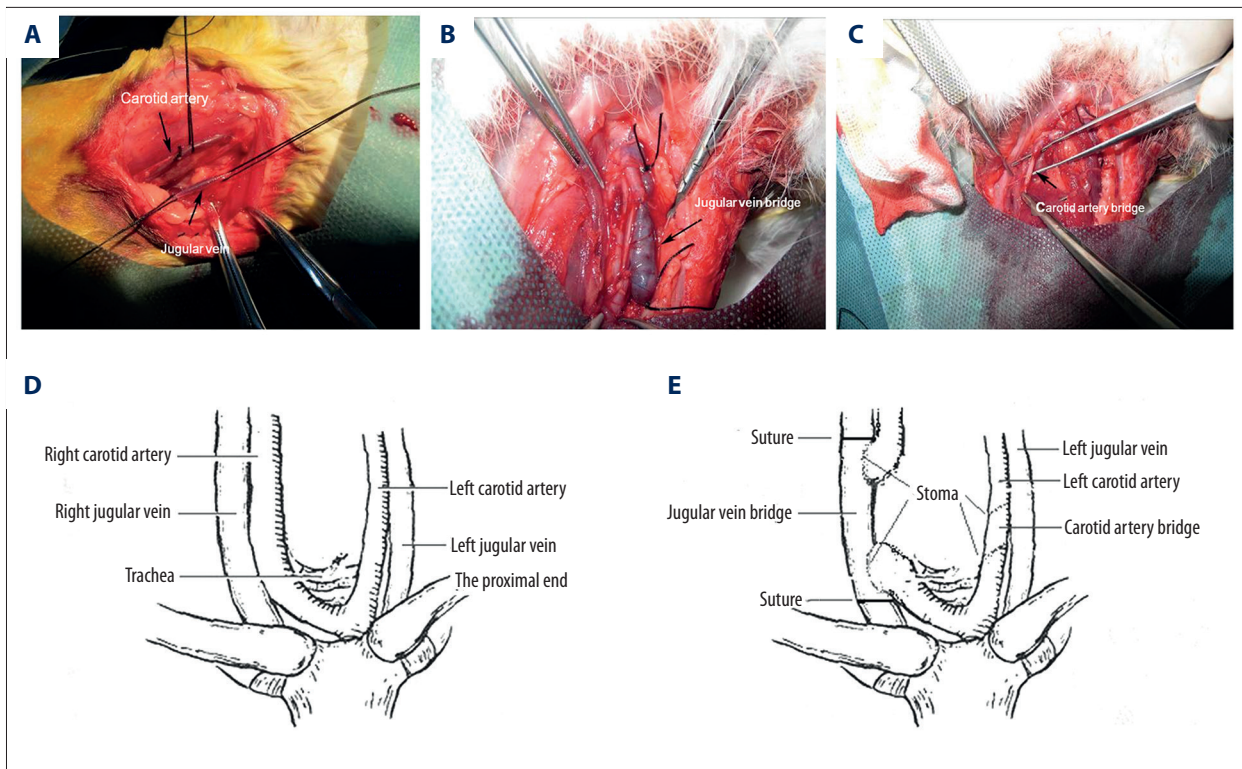
Figure 1. Representative pictures of surgery process and schematic diagram of grafting procedure. (**A**) Normal anatomy of rabbit carotid artery and jugular vein. (**B**) Construction of a right jugular vein bridge. (**C**) Construction of a left carotid artery bridge. (**D**) Schematic diagram of cervical blood vessels. (**E**) Schematic diagram of bilateral carotid artery grafting (ligature, venous bridge, ligature, anastomotic stoma, internal jugular vein, common carotid artery, and arterial bridge).

artery segment was connected to the left carotid artery via double end-to-end anastomosis followed by continuous suture of anastomotic stomas using an 8/0 Prolene suture. The left carotid artery was then released for air exhaust as described above. In this manner, a carotid artery bridge was built to bypass arterial blood flow from the proximal artery to the distal artery (Figure 1C). Schematic diagrams of the surgical procedures are illustrated in Figure 1D and 1E. Small intervals of carotid artery and jugular vein graft specimens were taken during surgery for morphological examinations.

Morphological examination

Pressure flush was performed with 10% paraformaldehyde to maintain the diameter of vascular specimens at their original levels prior to sampling. Specimens were subjected to conventional fixation, paraffin embedding, serial sectioning (slice thickness=0.4 µm), and hematoxylin and eosin staining. The thickness of the tunica intima and the inside areas of blood vessels were measured. Additionally, the rate of luminal stenosis was calculated using the following formula:

Rate of luminal stenosis (%) = (1 – residual luminal area/area below internal elastic lamina) ×100%.
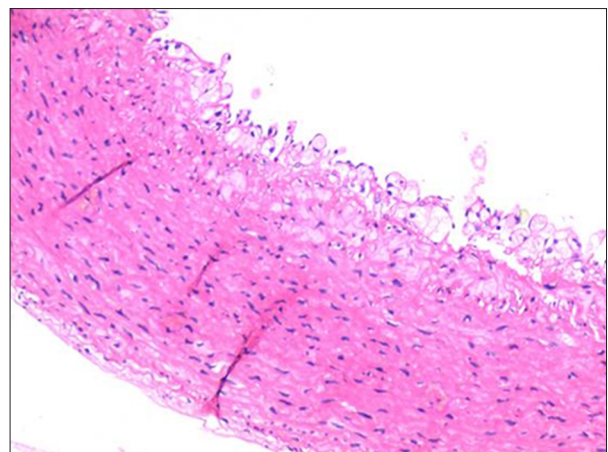

Figure 2. Histological image of the carotid artery in an animal model prior to surgery (8 weeks on a high-fat diet). Lipid deposition in the tunica intima and some liposome formation is clearly visible (hematoxylin and eosin staining, ×100).

Scanning electron microscopy (SEM) analysis

The sections of cervical blood vessels collected at different time intervals after surgery were rinsed with normal saline and fixed
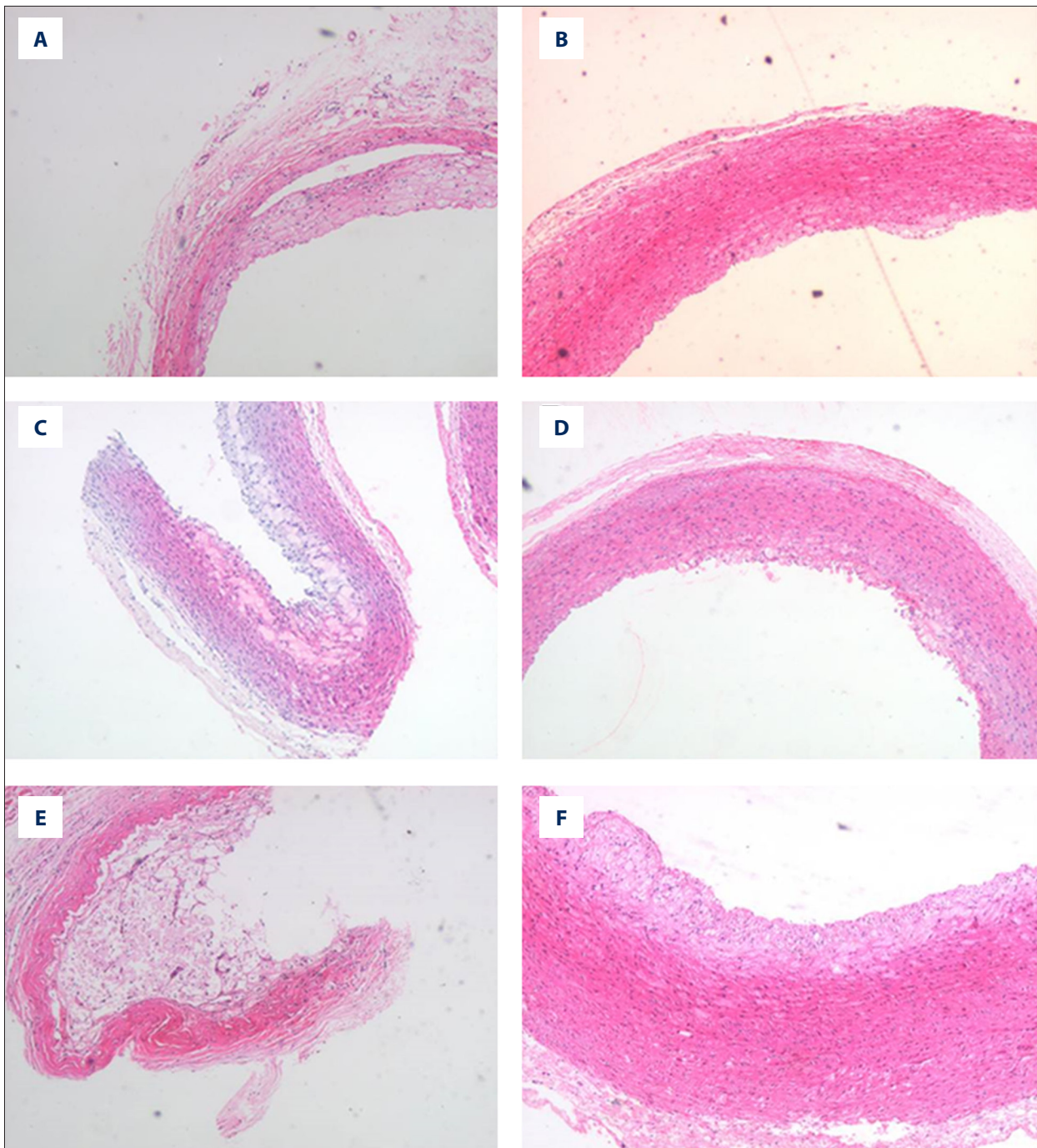
Figure 3. Histological images of jugular vein grafts (**A, C, E**) and carotid artery grafts (**B, D, F**) in our animal model. (**A, B**): 4 weeks after surgery (12 weeks on a high fat diet). (**C, D**): 8 weeks after surgery (16 weeks on a high-fat diet). (**E, F**): 12 weeks after surgery (20 weeks on a high-fat diet). Note the occurrence of intimal hyperplasia to a lesser extent in the carotid artery than in the jugular vein (hematoxylin and eosin staining, ×40).

in 2.5% glutaraldehyde on ice for 4 h. Following fixation, samples were thoroughly rinsed with 0.1 mM phosphate-buffered saline (PBS) 3 times for 10 min each. Specimens were fixed in the dark on ice in a 0.1-mM PBS solution containing 1% osmic acid for 1 h. After rinsing with double-distilled water, specimens

were immersed in 1% tannic acid solution for 3 h followed by an additional rinse in double-distilled water. Dehydration was performed in gradient ethanol (50%, 70%, 80%, 90%, and 100%, v/v) for 15 min each; the final dehydration step in 100% ethanol was performed twice. After freeze-drying, the specimens

were fixed on an objective table and coated with ion sputtering coating (approximately 10 nm). Finally, specimens were examined and photographed using SEM (Phenom G1, USA).

Statistical analysis

All statistical analyses were performed using SPSS Statistics 20.0 software (IBM, USA). All data are expressed as mean ± standard deviation. Differences between groups were compared with the *t* tests. *P*-values less than 0.05 were considered statistically significant.

Results

Atherosclerosis induced by high fat diet and associated pathological features

During surgery, a 0.3-cm carotid artery segment was taken for pathomorphological examination. Histological analysis revealed that after 8 weeks on a high-fat diet, only 1 animal had no noticeable atherosclerotic changes; in contrast, the remaining 19 animals had lipid deposition and partially formed liposomes in the tunica intima of carotid arteries, indicating the occurrence of atherosclerotic changes (Figure 2). Of the 19 animals with atherosclerotic changes, 1 died during surgery and the remaining 18 animals were used for additional analyses. We killed animals at 3 time points after surgery (12, 16, and 20 weeks; n=6 per time point). Blood vessel patency was maintained in all vascular graft specimens despite varying degrees of intimal hyperplasia and vascular stenosis.

Histomorphological changes in vascular graft restenosis

After 8 weeks of being fed a high-fat diet but prior to surgery, vascular media of the rabbit jugular vein was constituted with several layers of smooth muscle cells (SMCs) consisting of neatly arranged intimal cells. In contrast, the carotid artery contained more layers of SMCs and displayed mild intimal cell proliferation and lipid deposition.

Four weeks after surgery (a total of 12 weeks on a high-fat diet), endothelial cells began to fall off the vein grafts and some cells underwent proliferation accompanied by lipid deposition (Figure 3A); there were no significant changes in the artery graft (Figure 3B).

Eight weeks after surgery (16 weeks on a high fat diet), endothelial cells in the vein grafts began to recover and proliferate. There was also some lipid deposition and liposome formation in the tunica intima and partial mitigation of SMCs into the tunica intima (Figure 3C). Additionally, artery grafts exhibited focal proliferation of endothelial cells but not SMC mitigation (Figure 3D).
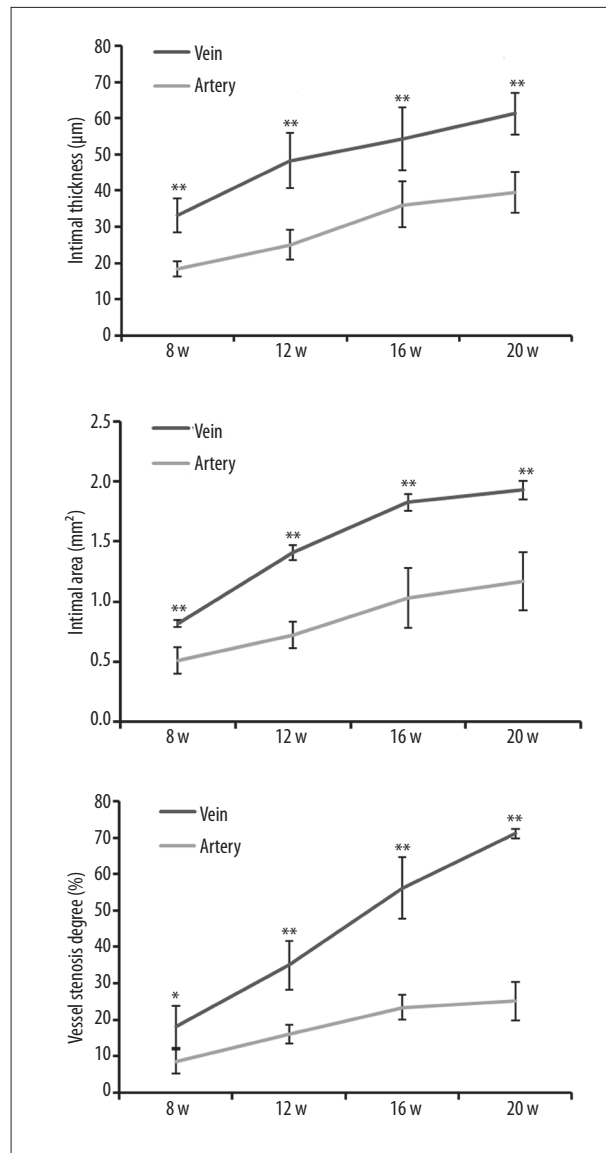

Figure 4. Variations in the degree of stenosis in jugular vein and carotid artery grafts in the rabbit model at different time points after surgery (n=6). * *P*<0.05, ** *P*<0.01.

Twelve weeks after surgery (20 weeks on a high-fat diet), endothelial cell repair within the vein graft was generally completed. This group showed the most significant proliferation and SMC migration, resulting in luminal stenosis of the grafted vessel (Figure 3E). Arterial grafts exhibited mild endothelial cell hyperplasia – to a lesser extent than vein grafts – with a smaller number of SMCs migrating into the tunica intima and causing circumscribed luminal stenosis (Figure 3F). The degrees of stenosis in jugular vein and carotid artery grafts were measured at different time intervals after surgery. It was evident that the initial stenosis and the progression of stenosis in vein grafts were more extensive than in artery grafts during the observation period (Week 20; Figure 4 and Table 1).

Table 1. Comparison of stenosis in the implanted veins and arteries of rabbits at various time points after operation (n=6, mean ±sd).

	Vein	Artery	t	P
Intimal thickness (µm)				
8 w	33.2±4.8	18.5±2.1	6.873	<0.01
12 w	48.3±7.6	25.1±4.2	6.545	<0.01
16 w	54.3±8.6	36.2±6.3	4.159	<0.01
20 w	61.3±5.8	39.6±5.6	6.593	<0.01
Intimal area (mm²)				
8 w	0.82±0.03	0.51±0.11	6.660	<0.01
12 w	1.41±0.06	0.72±0.11	13.489	<0.01
16 w	1.83±0.07	1.03±0.25	7.548	<0.01
20 w	1.93±0.08	1.17±0.24	7.359	<0.01
Vessel stenosis degree (%)				
8 w	18.1±5.7	8.5±3.3	3.570	<0.05
12 w	35.1±6.7	16.1±2.6	6.476	<0.01
16 w	56.2±8.5	23.4±3.4	8.776	<0.01
20 w	71.2±1.3	25.2±5.3	20.648	<0.01

Morphological changes in vascular graft restenosis detected by SEM

The internal elastic lamina is an elastic fiberboard that separates the tunica intima from the medial smooth muscle layer in the vascular wall. Prior to surgery (Week 8 of the experimental period), SEM analysis revealed that in both the control vein and artery, SMCs were located in the tunica media with a continuous internal elastic lamina. The medial SMCs appeared not to migrate toward the tunica intima. Moreover, liposomes formed in a portion of the tunica intima (Figure 5). After surgery (Week 20 of the experimental period), SEM analysis showed fracturing of the internal elastic lamina of the arterial graft. It also revealed some medial SMCs that had migrated into the tunica intima through the gap in the fractured internal elastic lamina. The fracture of the internal elastic lamina was more evident in the vein graft, where the complete structure of the internal elastic lamina was practically invisible. Additionally, many medial SMCs migrated into the tunica intima, and liposomes were phagocytosed by macrophages (Figure 6).

Discussion

In the current study, we induced atherosclerotic lesions in rabbits by feeding the animals a high-fat diet. We then simulated CABG, as it is conducted in human subjects, by performing bilateral carotid artery grafting using artery and vein grafts. This approach successfully recapitulated CABG in human subjects, and, thus, provided us with a robust model to examine mechanisms of restenosis in artery and vein grafts.

Formation of atherosclerotic lesions

We carried out a preliminary experiment in which animals were killed after being maintained on a high-fat diet for 4 weeks. However, pathomorphological examination of arterial specimens did not indicate any significant changes related to development of atherosclerosis (e.g., presence of liposomes and atherosclerotic plaques); instead, there was only evidence of some limited intimal hyperplasia. Next, we maintained animals on a high-fat diet for 8 weeks. In this case, pathomorphological examination revealed evidence for atherosclerosis in 19/20 animals; such evidence included liposome formation in diseased vessels, which was confirmed by lipid staining. Based on these data, we conclude that 8 weeks of a high-fat diet supplemented with high-dose vitamin D is a reliable method for inducing atherosclerotic lesions in rabbits.

Establishment of an animal model of bilateral carotid artery graft restenosis

New Zealand rabbits have coronary vessels that are small in diameter. Thus, we selected the carotid artery system, which
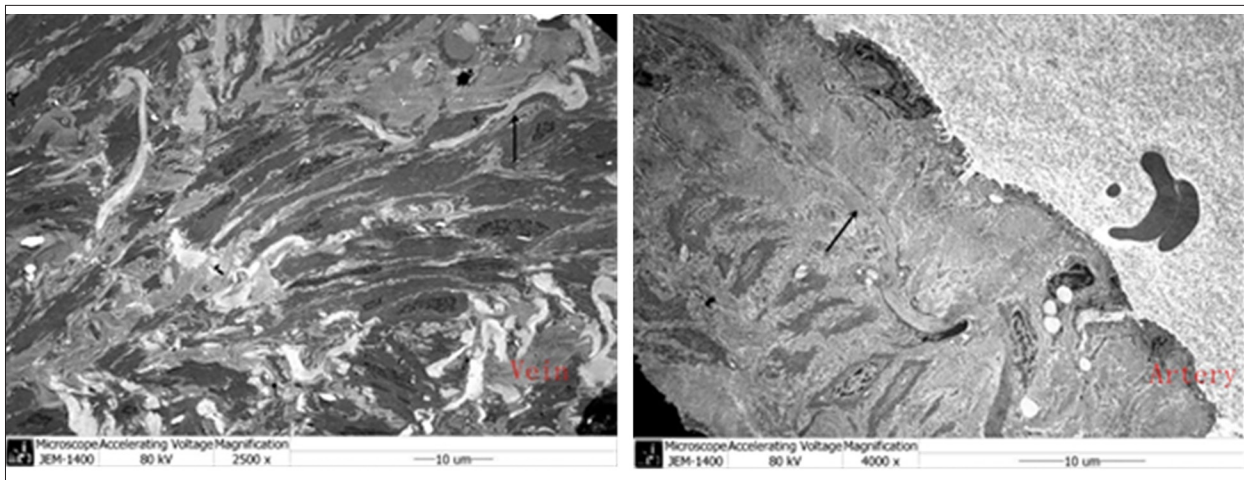
Figure 5. Scanning electron microscope analysis of morphological changes in the control jugular vein and carotid artery in our rabbit model before surgery (8 weeks on a high-fat diet; vein ×2500; artery ×4000). The images illustrate part of the cross-section of vascular tissue; arrows point to internal elastic lamina of the blood vessel.
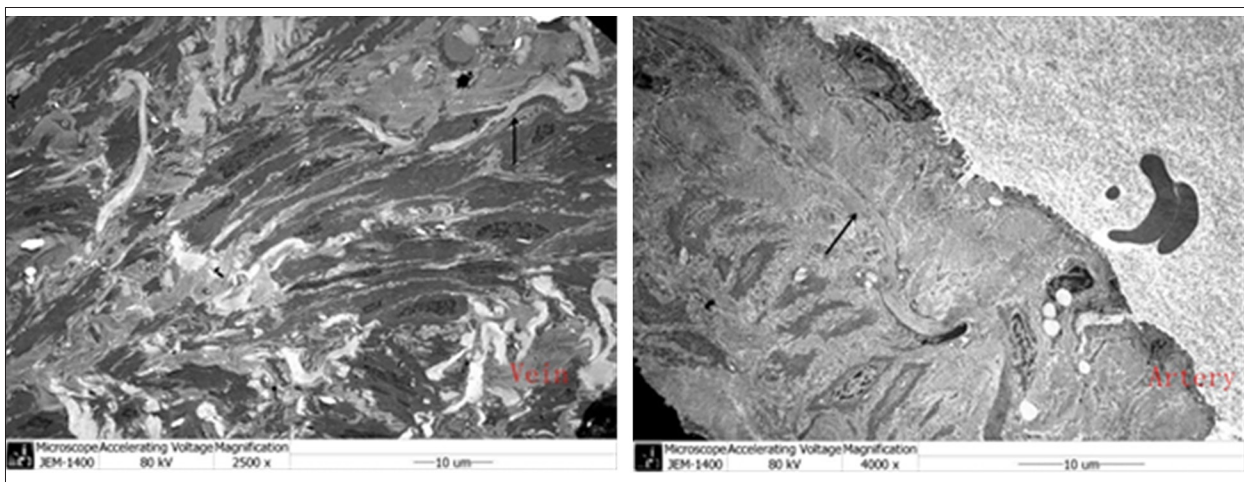

Figure 6. Scanning electron microscopy analysis of morphological changes in jugular vein and carotid artery grafts in our rabbit model after surgery (20 weeks on a high-fat diet; vein ×4000; artery ×3000). Note that the internal elastic lamina is fractured in vein and artery grafts. Moreover, some of the medial SMCs have migrated into the tunica intima through the fractured internal elastic lamina. The internal elastic lamina fracture was more evident in the vein graft than in the artery graft.

is prone to atherosclerosis, for the grafting of artery and vein bridges. After surgery, both bridge vessels were engorged, with sufficient distal pulsatility. Because the animals underwent bilateral vascular grafting in their carotid arteries, surgical failure or vascular occlusion would directly cause death of the experimental animals. Thus, this experiment has a high demand for surgical care and accuracy. Unfortunately, in the present study, 2 animals died at an early experimental stage due to failure of vascular anastomosis. Improved methods to ensure survival of all experimental animals after surgery would further illustrate the high degree of patency of the anastomotic stoma.

Selection of target vessels

The technique of vascular anastomosis first emerged at the beginning of the 20th century and matured into a developed, well established method by the mid-20th century. The methodology has continued to grow and evolve, and, in recent years, a variety of new vascular anastomosis procedures have been developed [7–9]. Vascular graft modeling in small animals facilitates *in vivo* studies of grafted vessels. Such models allow the study of complex pathological changes, the observation of patency rate, and in-depth molecular biological studies [10–13].

In the present study, we encountered a number of difficulties in establishing a useful animal model that could recapitulate

the human condition. The coronary vessels of New Zealand white rabbits have small diameters. Thus, surgical procedures are challenging and selection of the most appropriate target vessels for vascular grafting is key to the success of the procedure. Blood vessels deep within the muscles of limbs (e.g., femoral artery and vein) are closely associated with surrounding tissue and adjacent bones; moreover, the venous wall in small animals is relatively thin. Thus, even the smallest mistake could cause tearing and bleeding. This would make separating and preparing the blood vessel ends for anastomosis much more difficult. Once tearing and bleeding of the vein wall occurred, subsequent surgical procedures would be impossible.

We selected the rabbit carotid artery, quite prone to developing atherosclerosis, for the grafting of artery and vein bridges. Because superficial cervical tissues are exposed, they are subject to separation of cervical blood vessels. Additionally, there are carotid arteries at both sides of the cervical region, making it possible to perform concurrent artery and vein grafting within the same animal. Designing self-control parameters would further improve the comparability of the data.

Vascular anastomosis

Vascular grafting in small animals requires skill to perform anastomosis. Here, we followed the procedure of vascular anastomosis for human CABG. While constructing the vein graft, we sheared the carotid artery stump into a horn-like shape. Continuous suture was performed for end-to-side anastomosis of the carotid artery stump to the jugular vein. Modification of the carotid artery stump into a horn-like shape greatly increased the diameter of the anastomotic stoma and ensured the success of vascular anastomosis.

For end-to-end anastomosis of the arterial bridge, we cut the anastomotic stoma in an oblique section. The 2 vascular stumps to be anastomosed were fixed at the 6 and 12 o'clock positions. Continuous suturing was carried out from the posterior wall to the anterior wall. During suturing, the stitch length was approximately 0.5 mm. Excessively high stitch density would narrow the anastomotic stoma; in contrast, low stitch density would increase bleeding. Additional stitches can be added if bleeding occurs after the vascular graft is released. By practicing this anastomotic technique, surgeons can improve their suturing techniques in vascular surgery and familiarize themselves with microsurgical instruments and micro-vascular anastomotic techniques.

In the 1990s, Mizuta et al. [14] proposed the cuff technique for vascular anastomosis in experimental animals. We attempted to use this technique for anastomosis of vascular grafts in the preliminary experiment because of its low degree of surgical difficulty. In practice, however, we found large differences in blood vessel diameter between the jugular vein and carotid artery of New Zealand white rabbits (up to 2-fold). This precluded the use of the cuff technique for vascular grafting. Additionally, postoperative presence of foreign cuff material within the vascular grafts would negatively influence the experimental results. Thus, we chose to use the method of vascular anastomosis most closely related to that used in human CABG.

The main cause for vascular restenosis after CABG is excessive intimal thickening induced by SMC mitigation

Mast cells are an important source of vasocontractile and chemotactic substances, such as histamine, leukotriene C4, and platelet activating factor. Cross et al. identified mast cells in rabbit vein grafts 4 weeks postoperatively; no mast cells were found in ungrafted veins. In this study, vein grafts showed a contractile response to histamine, which was abolished by an H1 receptor antagonist [15]. Brauner et al. [16] found that 4 weeks post-surgery, vein grafts of hypercholesterolemic rabbits exhibited a significantly increased (contractile) sensitivity to serotonin and decreased (vasodilatory) sensitivity to sodium nitroprusside compared to vein grafts of normocholesterolemic rabbits. Additionally, hypercholesterolemic rabbits displayed increased neointimal hyperplasia. Vein grafts from these animals also showed lipid vacuoles and foam cells interspersed between the SMCs, which was supported by findings from another research group [16,17]. Following implantation, the vein graft, which is initially only subject to an internal pressure of 10 mmHg, is immediately subjected to arterial pressure (100 mmHg) and an immediate increase in flow, stress, and deformation. These forces may promote intimal hyperplasia. In our study, we identified hemodynamic changes in vein grafts after surgery. Due to arterial flow and shear stresses, endothelial cells were damaged, and intimal hyperplasia began to occur within the tunica intima 4 weeks after surgery (12 weeks on a high-fat diet). Thus, these findings are consistent with previous reports. Endometrial cell repair began by 8 weeks postsurgery and was completed by 12 weeks. Also by this time, mitigation of medial SMCs into the tunica intima occurred. Previous studies have demonstrated that the ratio of luminal radius to wall thickness in grafts tends to adapt to the same value as that in the grafted artery; suggesting that wall thickening occurs to normalize tangential wall stress [18]. Finally, in our study, the degrees of intimal hyperplasia and lipid deposition in artery grafts were lower than those in vein grafts.

In this study, we found that the degree of vascular stenosis was significantly higher in vein grafts than in artery grafts at all time points examined. This is consistent with other studies in small animal models showing that vein grafts are associated with better SMC intimal hyperplasia compared to artery grafts [19]. However, in clinical trials, differences between the use of artery grafts versus venous grafts do not seem to be as clear [20]. Thus, additional work needs to be performed to

better understand exactly how the use of small animal models translates to human subjects.

Conclusions

The method we propose here can be used to successfully establish an animal model of vascular restenosis in bilateral carotid artery grafting in rabbits. Our findings provide insight into the pathology of vein graft disease and allow us to test various therapeutic strategies *in vivo*.

Conflict of interest

None.

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